61. Method for the preparation of microbicidal peptides comprising adding a His-tag sequence to a peptide to produce a microbicidal peptide having enhanced activity in comparison to the same peptide without the additional His-tag sequence.

REMARKS

Claims 22-35 are pending in the application. Claims 22-35 have been cancelled. New claims 36-61 have been added. Support for the new claims can be found in the specification and drawings as originally filed. Reexamination and allowance of the claims are respectfully requested.

The Examiner has objected to the drawings for various informalities indicated on the Notice of Draftsperson's Patent Drawing Review dated August 22, 2000. The Applicants submit herewith corrected drawings of Figs. 1 - 13 correcting these informalities. Reconsideration of these objections is respectfully requested.

The Examiner has objected to the claims for not complying with the requirement of sequence identifiers as set forth in 37 CFR §1.821(d). The claims have been amended to supply sequence identifiers.

The Examiner has objected to the specification for not complying with the requirement of sequence identifiers as set forth in 37 CFR §1.821(d). The specification has been amended to supply sequence identifiers to accompany references to sequences in the text of the description.

The Examiner has rejected claim 22 and claims dependent therefrom under 35 U.S.C. § 102 for direction to non-statutory subject material. The new claims presented in this amendment specify that the peptide is isolated. Support is found for this characterization on page 2, line 6 of the specification. It is therefore believed that the rejection of claim 22 and dependent claims under 35 U.S.C. § 101 has been overcome.

The Examiner has rejected claims 33-35 under 35 U.S.C. § 101 for omitting the steps involved in a recited use. The new claims presented in this amendment are drawn to products or processes rather than to uses. It is therefore believed that the rejection of claims 33-35 under 35 U.S.C. § 101 has been overcome.

The Examiner has rejected claim 22 and claims dependent therefrom under 35 U.S.C. § 112, second paragraph, for indefiniteness. The Examiner asserts that it is unclear as to which specific arrangement claim 22 refers. The claims as amended refer to sequences identified by SEQ ID NO: and defined variations thereon.

The Examiner has rejected claims 28 and 29 under 35 U.S.C. § 112, second paragraph, for indefiniteness. The Examiner asserts that the phrase "for example" renders claims 28 and 29 indefinite. The corresponding claims presented in this amendment do not contain this phrase.

The Examiner has rejected claims 25 and 26 under 35 U.S.C. § 112, second paragraph, for indefiniteness. The Examiner asserts that the meaning of "variant" is unclear, and is in conflict with the recitation of a sequence. The corresponding claims presented in this amendment do not contain the word "variant."

The Examiner has rejected claims 22-27 under 35 U.S.C. § 102(b) for anticipation by U.S. Patent No. 5,656,724 to Daly et al., (hereinafter "Daly"). The Examiner asserts that Daly discloses recombinant CXC chemokines having amino acid sequences that comprise the exact same amino acid sequence of TC-1 and TC-2 of the present application. The Examiner also asserts that Daly describes these CXC chemokines as small inducible proteins having a specific arrangement of four position-invariant cysteine residues in their primary amino acid sequence that form two disulfide bonds. Applicants note that, though SEQ ID NO: 9 of Daly contains the peptides of SEQ ID NO: 12 (TC-1) of the present specification, and SEQ ID NO:

10 of Daly contains the peptides of SEQ ID NO: 6 (TC-2) of the present invention, none of the antimicrobial peptides of the present claims are disclosed in any of the cited documents.

Moreover, according to the present invention it has surprisingly been found that potent antimicrobial peptides, in particular peptides with a direct microbicidal activity, can be obtained by minor modifications of the well-known chemokines CTAP-III and NAP-2. TC-1* has, for example, been proven to be a potent microbicidal agent by deleting the two C-terminal amino acids of the chemokine NAP-2, whereas TC-2 has been proven to be a direct microbicidal agent only by deleting the two C-terminal amino acids of the chemokine CTAP-III.

Because these minor structural differences result in dramatic changes in activity of the compounds involved, the structures of NAP-2 and CTAP-III do not teach or suggest the structures of the claimed microbicidal peptides. Accordingly, the rejection of claims 22-27 over Daly is believed to have been overcome.

The Examiner has rejected claims 22-29 and 31-33 under 35 U.S.C. § 102(b) for anticipation by World Patent Document WO 99/06321 to Baggiolini et al. (hereinafter "Baggiolini"). The Examiner asserts that Baggiolini teaches the administration of daily doses of recombinant CXC chemokine NAP-2 for the treatment of human conditions associated with bacterial and fungal infections. The Examiner also asserts that NAP-2 is 97% identical to TC-1 in the present application, and 82% identical to TC-2 in the present application. The Examiner further asserts that the arrangement, disulfide linkages and three-dimensional structure disclosed in the present specification are inherent properties of this class of antimicrobial peptides, and that NAP-2 is a variant of TC-1 and TC-2, and anticipates the present invention.

As has been noted previously, the claims have been amended to present the claimed structures without the use of the term "variants." In addition, the peptides of the present invention do differ from other peptides in their class in three-dimensional structure and in other

properties. The peptides of the present invention have been shown to display a direct antimicrobial activity against a number of microorganisms (Example 3) by *in vitro* incubating bacteria with the thrombocidins. In contrast, the well-known chemokines exert their antimicrobial activity by complex interactions with other components of the immune system, such as leukocytes, and thus can only display their antimicrobial activity in the presence of a complete immune system. The well-known chemokines thus do not have microbicidal activity: the direct, or *in vitro*, antimicrobial activity described on p. 2, lines 12 of the specification and recited in claims 36-66 presented herewith.

It has also, unexpectedly, been found that the peptides according to the present invention do not need to be refolded after synthesis in order to display their direct antimicrobial activity, whereas refolding is necessary for chemokines to display their chemokine activity (page 13, lines 13-32). The peptides of the present invention can thus be produced recombinantly, in contrast to the well-known chemokines. Because cationic peptides, such as thrombocidins, generally act on the bacterial membrane itself, which cannot easily be modified, resistance of the bacteria against thrombocidins may not rapidly occur, and thus the peptides of the invention show great promise for use as new antimicrobial agents.

The peptides of the present invention differ from known chemokines in terms of reactivity as well as in terms of structure. The general antimicrobial activity of the known chemokines does not imply the use of peptides according to the present invention. It has been demonstrated in the present application (page 5, lines 11-15) that, with respect to endocarditis, in a platelet clot, bacteria are protected from phagocytic cells which cannot penetrate the dense platelet network, thus limiting the use of chemokines for treating such disorders. In contrast, the thrombocidins of the present invention are capable of penetrating the clot and thus may be effectively used to prevent bacterial proliferation.

For these reasons, the rejection of claims 22-29 and 31-33 is believed to have been overcome.

The Examiner has rejected claims 22, 32 and 34 under 35 U.S.C. §103(a) for purported obviousness over Baggiolini in view of Cimbollek et al., *Antimicrob. Agents Chemother.* **40**(6): 1432-7 (1996) (hereinafter "Cimbollek"). The Examiner asserts that Cimbollek teaches that both fungal and bacterial infections are associated with endocarditis. The Examiner concludes that it would have been obvious to a person skilled in the art to use the known antimicrobial chemokine NAP-2 for the treatment of endocarditis caused by fungal and bacterial infection.

As has been noted previously, the peptides of the present invention differ from those disclosed in the cited prior art in terms of structure and properties. The peptides of the present invention display a direct antimicrobial activity against a number of microorganisms; the chemokines of the prior art act indirectly, in concert with other components of the immune system. The peptides according to the present invention do not need to be refolded after synthesis in order to display their direct antimicrobial activity, whereas refolding is necessary for chemokines to display their chemokine activity. The peptides of the present invention can penetrate a platelet network to prevent bacterial proliferation; the known chemokines are not able to do so. The antimicrobial properties of chemokine NAP-2 therefore do not teach or suggest its use for the treatment of endocarditis caused by fungal and bacterial infection. The new claims presented in this Amendment recite the microbicidal properties of the claimed species; the chemokines of the prior art do not possess these properties, and the cited references do not teach or suggest them. For these reasons, the rejection of claims 22, 32 and 34 over Baggiolini in view of Cimbollek is believed to have been overcome.

The Examiner has rejected claims 22, 30 and 35 under 35 U.S.C. §103(a) for purported obviousness over Baggiolini in view of U.S. Patent No. 4,725,576 to Pollock et al.

(hereinafter "Pollock") and further in view of U.S. Patent No. 5,073,627 to Benson et al. (hereinafter "Benson"). The Examiner asserts that Pollock teaches the antimicrobial effects of L-histidine and histidine-rich polypeptides, specifically the antimicrobial effects of His-7 and His-4, containing 4 and 7 residues of L-histidine, respectively. The Examiner also asserts that Benson teaches a fusion protein made by combining GM-CSF and IL-3, that GM-CSF and IL-3 have considerable overlap in their range of biological activities, and that the fusion protein has enhanced biological activity when compared to IL-3 or GM-CSF alone. The Examiner concludes that it would have been obvious to a person skilled in the art to fuse a Histag, or His-6, to the NAP-2, a known CXC chemokine having a specific arrangement and disulfide linkage of 4 cysteine residues, in order to achieve enhanced activity.

However, this combination does not teach or suggest the present invention for a number of reasons. The use of NAP-2, or even the use of chemokines in general, does not teach or suggest the use of the polypeptides of the present invention, because of structural and reactivity differences between prior art chemokines and the peptides of the present invention, as explained previously. In addition, according to the present invention, the addition of the Histag enhances further the unexpected direct antimicrobial, i.e., microbicidal, activity of the novel and inventive peptides of the present invention. None of the cited references teaches this unexpected activity. Because of this difference in modes of actions between the prior art and the present invention, the teachings of Baggiolini, Pollack and Benson, either alone or in combination, do not make it obvious that the addition of a Histag necessarily results in increased activity in the present invention. For these reasons, the rejection of claims 22, 30 and 35 over Baggiolini in view of Pollock and Benson is believed to have been overcome.

In view of the above amendments and remarks, it is believed that the claims are in condition for allowance. Reconsideration of the rejections is requested. Allowance of claims 36-61 is respectfully requested.

Respectfully submitted,

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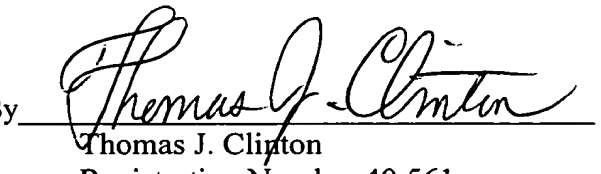
By 

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MARKED-UP VERSION OF THE SPECIFICATION

On page 1, please delete and replace the current version of the sixth full paragraph starting on line 31 and bridging page 2, ending on line 7, with the following replacement paragraph:

This object is achieved by the invention by providing new, isolated or recombinant, antimicrobial peptides thrombocidin-1 (TC-1) **(SEQ ID NO: 12)** and thrombocidin-2 (TC-2) **(SEQ ID NO: 6)** or variants thereof, such as TC-1*, **(SEQ ID NO: 3)**, which comprise, at least in part, the sequence as shown in figure 1 and have broad antimicrobial activity. These peptides, or variants thereof, thus may be effectively used as antibiotics in the treatment of several infectious diseases. These peptides can be isolated from both human and animal tissue.

On page 3, please delete and replace the current version of the first full paragraph starting on line 10 with the following replacement paragraph:

The new peptides of the invention appear to be derivatives of NAP-2 and CTAP-III. NAP-2 itself is a N-terminal cleavage product of CTAP-III. TC-1 has been shown to be a mixture of C-terminal truncation products of NAP-2, of which the 7436 Da peptide, lacking two C-terminal amino acids, is the main component (referred to as variant TC-1*;**(SEQ ID NO: 3)** figure 1, table 1). A form of NAP-2 with an additional N-terminal tyrosine was also present as a minor component. TC-2 **(SEQ ID NO: 2)** has been identified as a C-terminal truncation product of CTAP-III **(SEQ ID NO: 1)** lacking the last two C-terminal amino acids, with a molecular weight of 9100 (figure 1A, table 1). Thrombocidins identified thus far are indicated in fig. 1A, together with the known sequences of CTAP-III **(SEQ ID NO: 1)** and NAP-2 **(SEQ ID NO: 13)** (fig. 1).

On page 5, please delete and replace the current version of the third full paragraph starting on line 21 with the following replacement paragraph:

The present invention thus provides new, isolated or recombinantly prepared peptides TC-1 **(SEQ ID NO: 12)** and TC-2 **(SEQ ID NO: 6)**, or variants thereof, such as TC-1* **(SEQ ID NO: 3)** (fig 1 and 2), which exhibit antibacterial and/or antifungal activity and can be used in the treatment of infections in humans and animals. Furthermore, the peptides, or variants thereof, of the present invention can be used for the preparation of a medicament for the treatment of bacterial and/or fungal infections.

On page 8, please delete and replace the current version of the paragraph starting on line 37 and bridging page 9, ending on line 11, with the following replacement paragraph:

ES spectroscopy of TC-2 (fig 7b) yielded a molecular weight of 9100,5 ± 1,3. This value was confirmed by MALDI-tof spectroscopy. In addition to TC-2, only one minor contamination was present (10081 Da, fig 9). Partial sequencing of TC-2 indicated that the N-terminus of TC-2 is identical to that of CTAP-III. Based on the mass-spectrometrical data (figs 7b and 9) however, it appears that the mass found experimentally was smaller than the mass of CTAP-III (table 1). This can be explained by assuming that TC-2 is truncated C-terminally and misses 2 amino acids compared to CTAP-III. Thrombocidins identified thus far are indicated in fig 1, together with the sequences of CTAP-III **(SEQ ID NO: 1)** and NAP-2 **(SEQ ID NO: 13)**.

On page 10, please delete and replace the current version of the paragraph entitled EXAMPLE 2 that starts on line 1 and bridges page 11, ending on line6, with the following replacement paragraph: (Please note that both the marked-up version and the clean version contain underlining that is to be retained and does not constitute an amendment.)

EXAMPLE 2

Production of recombinant (r) CTAP-III, rNAP-2, rTC-1, rTC-1* and rTC-2.

From a human bone marrow CDNA library (Clontech, Palo Alto, USA) DNA coding for PBP was amplified in a PCR. 5' TATAGGATCCATGAGCCTCAGACTTGATAC CACC-3' **(SEQ ID NO: 7)** and 5' TATAGGATCC**TCA**ATCAGCAGATTCATCAC CTGCCAAT-3' **(SEQ ID NO: 8)** were used as forward and reverse primers, respectively. BamHI restriction sites (underlined) were added to allow cloning in a suitable vector. A stop sequence (boldface) was included to allow proper transcription termination at the stage of protein expression. This PCR was performed using 2 ng of template DNA and Pfu DNA polymerase, which has proofreading capacity. The resulting product was of the expected size (400 bp). This product served as a template in a second PCR to produce the coding DNA of TC-1 **(SEQ ID NO: 12)**, TC-2, CTAP-III **(SEQ ID NO: 1)**, NAP-2 **(SEQ ID NO: 13)** and TC-1* **(SEQ ID NO: 3)**, a variant of TC-1 which lacks two C-terminal amino acids (Ala-Asp) and carries two additional N-terminal amino acids (Ala-Glu) (fig 2). These PCR products were cloned into expression vectors. For CTAP-III, NAP-2 and TC-1 the reverse primer was the same as the reverse primer described above. The forward primers were as follows:

for CTAP-III and TC-2:

5' GTGTAACATATGAACTTGGCGAAAGGCAAAGAG-3' **(SEQ ID NO: 9)**;

for NAP-2 and TC-1*;

5' GTGTAACATATG**TA**TGCTGAACTCCGCTGCATG 3' **(SEQ ID NO: 10)**;

and for TC-1:

5' GTGTAACATATG**TAT**CTCCGCTGCATGTGTATAAAG-3' **(SEQ ID NO: 11)**.